PREPARATION OF STABILIZED ESSENTIAL FATTY ACID ENRICHED FORMULATIONS FROM MARINE SOURCES FOR USE AS NUTRACEUTICAL AND AQUAFEED SUPPLEMENTS

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The present investigation entitled **"PREPARATION OF STABILIZED ESSENTIAL FATTY ACID ENRICHED FORMULATIONS FROM MARINE SOURCES FOR USE AS NUTRACEUTICAL AND AQUAFEED SUPPLEMENTS"** has been discussed under different sections:

The initial sections explained the Acknowledgements, Abbreviations, Contents Abstract, List of tables, and List of figures. The eleven chapters followed by references & list of publications are as follows:

CHAPTER 1: INTRODUCTION

This is an introductory chapter and dealt with the importance of the present work. This chapter was further sub-divided into following sub-sections: section 1.1, which gave and introduction on polyunsaturated fatty acids (PUFAs); section 1.2, which explained the health benefits of essential PUFAs; section 1.3, which explained the essential polyunsaturated fatty acids and human diet; section 1.4, which explained the importance of marine fishes as a source of essential polyunsaturated fatty acids; section 1.5, which explained the importance of purified form of *n*-3 fatty acids; section 1.6 detailed the importance to the optimize the stabilization of fish oil against lipid oxidation; section 1.7, that discussed the relevance of the antioxidants to stabilize fish oil, and section 1.8 that elaborated the importance of the bioactivity enrichment of the purified fish oil.

Based on the above background the objectives of the thesis are as follows:

- To screen various marine fishes for higher content of polyunsaturated fatty acids (PUFAs).
- To prepare the fatty acid concentrate(s) by chemical hydrolysis of triglycerides from the selected marine source with high *n*-3 PUFAs followed by various physico-chemical/biotechnological procedures like amide complexation, enzymatic hydrolysis and/or chromatography based on differences in physico-chemical properties associated with the number of olefinic double bonds or acyl chain length.
- To study the shelf life of the refined/concentrated/purified PUFAs with regard to lipid peroxidation status, and reactive oxygen species (ROS) scavenging activity.
- To purify and structurally characterize components with antioxidant activities from different natural sources, especially marine sources and to shortlist potential antioxidant molecules/components.

- To prepare stabilized PUFA concentrate(s) by the addition of individual or combination of different selected antioxidant ingredients with potential antioxidant properties in an attempt to impart stability for health food/aquafeed supplements.
- To enrich the bioactivity with respect to anti-inflammatory property of the purified fish methyl esters using marine bioactive ingredients.
- To prepare a formulation containing purified PUFA esters and suitable additives for use in food applications as nutraceuticals, including dietary supplements, and animal/aquafeed products.

Based on the above objectives the present thesis is crystallized into a total of eleven chapters. The background and importance of the study with objectives are discussed and explained in the Introduction under Chapter 1. Chapter 2 deals with the detailed review of the works carried out regarding the significance of fish oil, chemistry and composition of marine oils, applications and uses of fish oils, processing of fish oils, degradation of fish oils, analysis of lipid oxidations, how to control their oxidative degradation, selection of antioxidants and enrichment of fish oils. Subsequently, the screening of marine fishes based on their seasonal variation in fatty acid composition and selection of the best species is described in Chapter 3. Chapter 4 describes the extraction and refining of triacylglycerides from Indian Oil Sardine S*ardinella longiceps*. The processing of the refined fatty acid to their purified methyl ester form is detailed in Chapter 5. In order to stabilize the purified oil selected marine additives were studied for their antioxidant activities. The bioactivity profile and the preparation of these additives are detailed in Chapter 6. The effects of antioxidant additives on the shelf-life of the refined oil, concentrated and purified methyl esters are described in Chapter 7, which also compared the antioxidant activities of the marine additives and natural herbs to prevent the oxidative degradation of the purified fish oil. In Chapter 8, the isolation and characterization of bioactive secondary metabolites from potential marine additives, which found to be the most effective additives for imparting oxidative stability to the sardine oil/esters, were evaluated. To further enhance the bioactivity of the purified methyl esters, different marine cephalopod extracts were prepared from different sources and a potent species based on their anti-inflammatory profile was short-listed and detailed in Chapter 9. An enriched PUFA formulation for use as nutraceutical and aquafeed supplements was prepared and their studies were described in Chapter 10. Chapter 11 summarized the entire work carried out in the present study.

CHAPTER 2: REVIEW OF LITERATURE

This chapter dealt with the overview of previous studies on chemistry and composition of fish oils, applications of fish oil, general overview of fatty acid synthesis and catabolism, metabolic pathway of *n*-3 fatty acids, extraction and refining of fish oils, purification of fish oils, oxidation of fish oils and antioxidant and pharmacologically active compounds from marine ecosystem.

CHAPTER 3: SCREENING AND SELECTION OF MARINE FISHES FOR POLYUNSATURATED FATTY ACIDS

3.1. Materials and Methods

3.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals and reagents used in this chapter and all the analytical instruments used in this chapter.

3.1.2. Samples and study area

The marine finfishes, *Trichiurus lepturus* (ribbon fishes), *Leiognathus splendens* (silver bellies), *Sardinella longiceps* (oil sardines) and *Epinephelus diacanthus* (spinycheek groupers) were collected from both Southwest (SW) and South east (SE) coast of India.

3.1.3. Lipid and Fatty acid Profile of the Candidate Marine Fishes

This section detailed the (3.1.3.1) Estimation of total lipids (3.1.3.2) Fatty acid profiling by gas-chromatographic analysis and (3.1.3.3) Evaluation of fatty acid based nutritional indices fatty acid profiling using by gas chromatography. The fatty acid based nutritional indices, mainly; atherogenicity (AI) and thrombogenicity (TI) indices and hypocholesterolaemic/ hypercholesterolaemic (HH) ratio, of these fishes were also calculated.

3.1.4. Nutritional Composition of *Sardinella longiceps*

This section dealt with the analysis of proximate composition (3.1.4.1), amino acids (3.1.4.2), vitamins (3.1.4.3) and minerals (3.1.4.4) of *Sardinella longiceps*.

3.2. Results and Discussion

This section detailed the results and discussion of this chapter and demonstrated that *Sardinella longiceps* is a valuable source of long-chain polyunsaturated fatty acids (PUFAs), especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), for human diet as well as the extraction of oil in view of commercial exploitation. It has been demonstrated that *Sardinella longiceps* harvested from the Southwest coast, especially

during monsoon/post-monsoon, showed its superiority over those harvested from the Southeast coast when EPA, DHA, total polyunsaturated fatty acids and all other fatty acid based nutritional indices were considered. Insignificant variations in fatty acid based indices, higher lipid yield, maximum EPA content and low saturated fatty acids for the oil sardines collected during the monsoon season (Southwest coast) compared to the oil sardines collected during post-monsoon have been noted. This demonstrated the importance of this low-value fish collected during monsoon season from the Southwest coast for commercial use, particularly for marine fish oil industries. This chapter also showed this species collected from the Southwest coasts during the monsoon season as a source of ideal nutrition due to the optimum balance of the essential nutrients *viz*., amino acids, minerals and vitamins. It is therefore, *Sardinella longiceps* collected during the monsoon season from Southwest coast can be considered as ideal raw material for further extraction, refinement and purification of polyunsaturated fatty acids as described in the following chapters.

CHAPTER 4: EXTRACTION AND REFINING OF TRIACYLGLYCERIDES FROM S*ARDINELLA LONGICEPS*

4.1. Materials and Methods

4.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals and reagents used in this chapter and the analytical instruments used.

4.1.2. Comparison of different triacylglyceride extraction techniques

This section detailed the different extraction methodologies of triacylglycerides (TAGs) from the edible tissues of *Sardinella longiceps* using (E_1) $CHCl_3$: MeOH; (E_2) 40% EtOH; (E_3) 100% EtOH (E_4) 100% H_2O (E_5) cooking followed by extraction with $CHCl_3$: MeOH (E_6) Cooking and pressing. The oil quality parameters have been analyzed to compare different lipid extraction techniques.

4.1.3. Refining of crude triacylglycerides

The crude triacylglycerides (TAG) obtained using cold pressing (E_6) was refined according to different protocols as discussed under the following sections.

4.1.3.1. Degumming and Neutralization of Crude Sardine Oil

The triacylglycerides (TAGs) obtained using cold pressing method (E_6) was degummed to remove the majority of phospholipidic compounds, by the addition of

different agents– (1) phosphoric acid (85%; 0.2% w/w; DG_1); (2) acetic acid (glacial; 0.2% w/w; DG_2); (3) oxalic acid (0.2% w/w; DG_3); (4) citric acid (0.2% w/w; DG_4). The degummed and neutralized sardine oil was studied for oil quality parameters. The observed optimum methodologies of degumming (DG_1 and DG_3) were further evaluated by detailed spectroscopic analysis. The TAGs obtained after degumming with phosphoric acid were further refined by caustic neutralization in order to obtain the neutralized sardine oil.

4.1.3.2. Bleaching of Neutralized Sardine Oil

This section detailed the methodology in neutralized sardine oil using different adsorbents such as activated charcoal (AC) powder, kaolin light (KL), fuller's earth (FE), cellulose powder, chitin purified powder. The five experimental combinations (at 5% w/w of oil) of the optimum adsorbents selected ie., AC and FE were, (BC_1) AC + FE (90:10), (BC_2) AC + FE (75:25), (BC_3) AC + FE (50:50), (BC_4) AC + FE (25:75), and (BC_5) AC + FE (10:90). The effect of the best adsorbent combination, i.e., (BC_4), AC + FE (25:75) was further studied for optimizing two experimental factors, *viz*., adsorbent amount and contact time of bleaching as shown in Table 4.1.

Table 4.1 Adsorbent amount and contact time of bleaching

	Adsorbent amount AC:FE (g in 100g oil)	Contact time (min)
BC_{4A}	5	20
BC_{4B}	5	40
BC_{4C}	5	60
BC_{4D}	3	20
BC_{4E}	3	40
BC_{4F}	3	60
BC_{4G}	1	20
BC_{4H}	1	40
BC_{4I}	1	60

Adsorbent amount 5% - Activated Charcoal (1.25 g) + Fuller's earth (3.75 g)
Adsorbent amount 3% - Activated Charcoal (0.75 g) + Fuller's earth (2.25 g)
Adsorbent amount 1% - Activated Charcoal (0.25 g) + Fuller's earth (0.75 g)
Contact time implies the time used for stirring the oil with the adsorbent combination by heating at 60 °C.

The optimum conditions were found out by analyzing the quality parameters. The bleached sardine oil obtained by the optimized bleaching condition, i.e., [(BC_{4E}; adsorbent amount (3%) and contact time (40 min)], was further assayed by ^{1}H NMR analysis.

4.1.3.3. Deodorization of Bleached Sardine Oil

The deodorization of the bleached oil (BC_{4E}) was carried out using a laboratory distillation unit. The boiling flask containing the bleached oil with aqueous acid solution

mixture (0.25N acetic acid) was heated to 100 ^{o}C under vacuum (5 mm Hg) for different time such as, 5 min (DO-01), 15 min (DO-02), 30 min (DO-03), and 60 min (DO-04). The volatile compounds collected at different intervals were analyzed using the GC-MS analysis.

4.1.4. Oil quality parameters

The protocols for analyzing the 4.1.4.1. Specific Gravity, 4.1.4.2. Free Fatty Acid Value, 4.1.4.3. Saponification Value, 4.1.4.4. Iodine Value, 4.1.4.5. Peroxide Value (P.V.), 4.1.4.6. The para-Anisidine Value (pA.V.), 4.1.4.7. Total Oxidation (TOTOX) Value, 4.1.4.8. Thiobarbituric Acid Reactive Species (TBARS) Value, 4.1.4.9. Rancimat Analysis, 4.1.4.10. Lovibond Color, 4.1.4.11. Ultraviolet Absorption, 4.1.4.12. Fatty Acid Profiling, 4.1.4.13. Gas Chromatography-Mass Spectrometry (GC/MS) analysis of fatty acid methyl esters (FAMEs) derived from optimum extraction mode, 4.1.4.14. NMR Analysis, 4.1.4.15. FTIR (Fourier Transform Infrared Spectroscopy), 4.1.4.16. GC-MS Analysis of the Volatile Components Obtained during Deodorization and 4.1.5. Statistical Analysis were discussed in the respective sections.

4.2. Results and Discussion

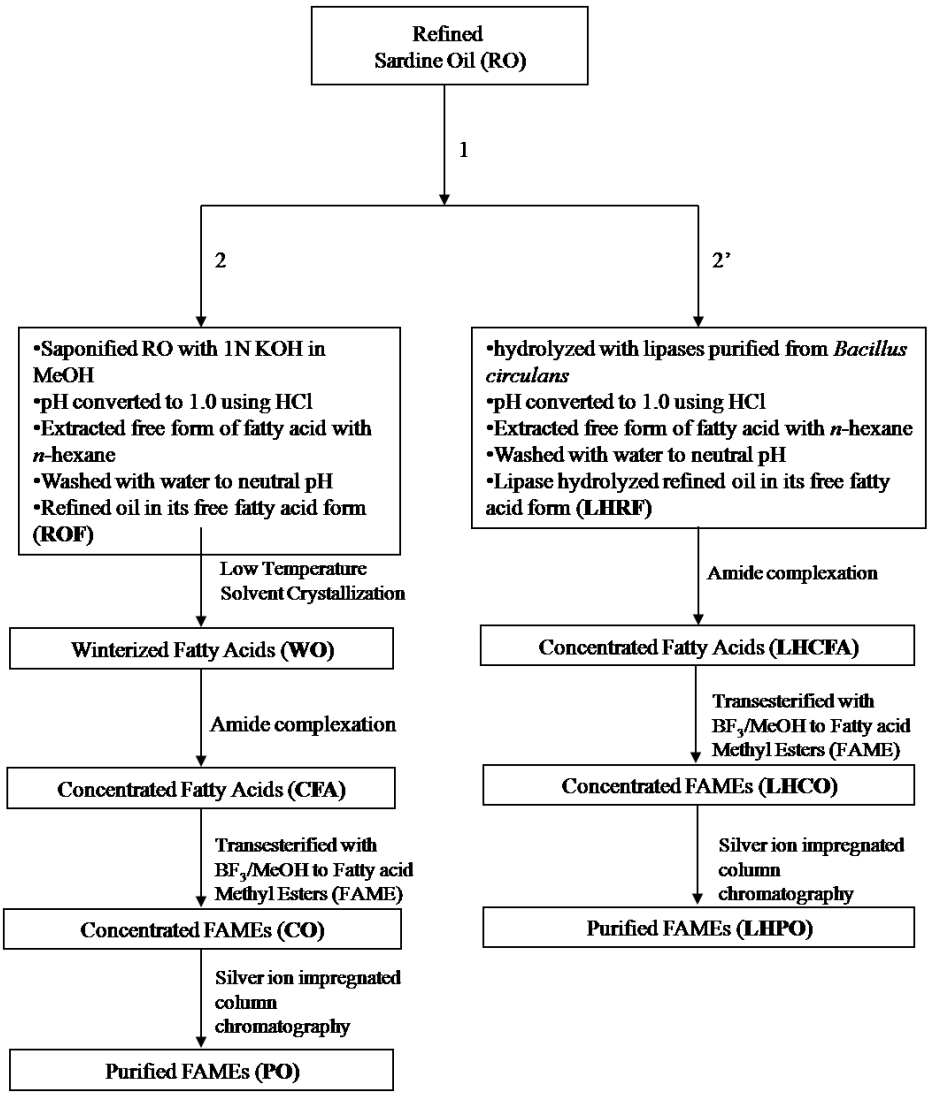
This section detailed the results and discussion of this chapter and concluded that the extraction of the oil using the process of cooking followed by pressing have been demonstrated to be important to obtain the good quality oil with respect to total yield and *n*-3 fatty acid composition such as EPA (20:5*n*-3) and DHA (22:6*n*-3). The quality parameters including the lipid oxidation parameters have been taken into consideration to assess the characteristics of the crude oil. Refining of the oil by degumming, bleaching and deodorization were effective in the removal of impurities such as the colored and volatile compounds. The effective degumming agent was found to be 1% phosphoric acid. The most effective adsorbent combination to carry out the bleaching process of degummed oil was found to be activated charcoal: Fuller's Earth at the ratio 1.25:3.75 (3% w/w ie. 0.75 + 2.25 g in 100 g oil). This study has demonstrated that unfavorable fishy odors can successfully be removed by distillation with aqueous acid solution mixture (0.25N acetic acid) (100 ^{o}C) under vacuum. The odor-contributing volatile components may be used as

flavor quality markers in the deodorization processes leading to food grade oil as determined by the GC-MS analysis. The study also demonstrated the importance of employing the spectroscopic techniques to assess the refining process of the crude oil. The region between δ 4-4.5 ppm in the ^{1}H-NMR spectra was taken into account to explain the bleaching process of the oil, and the signals at δ1.8 – 3.0 ppm (methine and acetyl), δ 3.0 – 4.5 ppm (free amine and hydroxyl signals), and δ 4.5 – 6.5 ppm (olefinic protons) were considered as the indicator region to explain the variability of the oil qualities under different treatment combinations during the degumming process. It was found that there was a significant reduction in the content of unsaturation (as in δ 4-5 ppm) while using activated charcoal: Fuller's Earth at the ratio of 1.25:3.75 (3% concentration) during the bleaching process. Significant reduction in the contents of hydrocarbon functionalities (as characterized by the decrease in the proton integral) was apparent by using 1% phosphoric acid during the course of degumming process. This study demonstrated that the degumming process by using phosphoric acid (1%) followed by bleaching by using the adsorbent combination of activated charcoal- Fuller's Earth (1.25:3.75, w/w, 3%), and deodorization by using distillation for one hour could be effective to afford a refined sardine oil with superior quality

CHAPTER 5: PREPARATION OF PURIFIED ESSENTIAL FATTY ACIDS

Background

This chapter highlighted the methodologies for purification of PUFAs from refined sardine oil with the goal to get a PUFA with high eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3). A schematic diagram showing the overall process of purification is shown below.


Refined
Sardine Oil (RO)
1
2
2'
•Saponified RO with 1N KOH in MeOH
•pH converted to 1.0 using HCl
•Extracted free form of fatty acid with n-hexane
•Washed with water to neutral pH
•Refined oil in its free fatty acid form (ROF)
•hydrolyzed with lipases purified from Bacillus circulans
•pH converted to 1.0 using HCl
•Extracted free form of fatty acid with n-hexane
•Washed with water to neutral pH
•Lipase hydrolyzed refined oil in its free fatty acid form (LHRF)
Low Temperature Solvent Crystallization
Amide complexation
Winterized Fatty Acids (WO)
Concentrated Fatty Acids (LHCFA)
Amide complexation
Transesterified with BF3/MeOH to Fatty acid Methyl Esters (FAME)
Concentrated Fatty Acids (CFA)
Concentrated FAMEs (LHCO)
Transesterified with BF3/MeOH to Fatty acid Methyl Esters (FAME)
Silver ion impregnated column chromatography
Concentrated FAMEs (CO)
Purified FAMEs (LHPO)
Silver ion impregnated column chromatography
Purified FAMEs (PO)

5.1. Materials and Methods

5.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals and reagents used in this chapter and the analytical instruments used in this chapter.

5.1.2. Saponification and Extraction of Free Fatty Acids from Refined Sardine Oil

About 100 g refined sardine oil (RO; *Chapter 4*) was saponified by refluxing with 500 ml of alkaline KOH solution (1N in MeOH) under continuous stirring for 45 min in an inert atmosphere of N_2 and are detailed in this section.

5.1.3. Preparation of purified essential fatty acids using physical and chemical techniques

The essential fatty acids in the refined sardine oil (RO) was purified by employing winterization (physical), urea complexation (chemical) and argentation chromatographic

(chemical) techniques. The techniques used were detailed in the sections as: (5.1.3.1.) Winterization of Refined Sardine Oil (5.1.3.2) Concentration of winterized, refined sardine oil (WO); (5.1.3.3) Acid-catalyzed trans-esterification of concentrated sardine oil (CO) to fatty acid methyl esters; (5.1.3.4.) Purification of concentrated fatty acid methyl esters (CO) by argentated chromatography. The optimum methodology to get maximum purity of *n*-3 fatty acids has been selected by assaying its fatty acid composition and the quality parameters. The purity of the essential fatty acids after each methods has been further validated using FTIR and NMR analysis. The argentated chromatographic purification has been detailed in the sub-sections as 5.1.3.4A. Preparation of Silver Impregnated Alumina or Silica, 5.1.3.4B. Argentation Chromatography using Ag-Alumina, 5.1.3.4C. Argentation Chromatography using Ag-Silica and 5.1.3.4D. Stirring with Ag-silica.

5.1.4. Preparation of purified essential fatty acids using physical, biotechnological and chemical techniques

The crude triacylglycerides of *S. longiceps* obtained by cold pressing (E6) was purified by employing winterization (physical), biotechnological (enzymatic hydrolysis) and chemical (urea complexation and argentated chromatography) techniques and detailed in the subsections., (5.1.4.1.) Isolation, production, purification and molecular weight determination of lipase from *Bacillus circulans* (5.1.4.1A.) This section details the isolation of *Bacillus circulans* from *Turbinaria conoides* and lipase production; (5.1.4.1B.) This section details the purification of lipase from *Bacillus circulans* by ammonium sulphated precipitation and chromatography; (5.1.4.1B.) This section details the determination of molecular weight of the lipase by denaturing polyacrylamide gel electrophoresis; (5.1.4.2.) lipase-catalyzed hydrolysis of the refined sardine oil by time-course (1–6 h) *Bacillus circulans* lipase-catalyzed hydrolysis and (5.1.4.3.) concentration and purification of lipase hydrolyzed sardine fatty acids (LHRF). The lipase hydrolyzed sardine fatty acids (LHRF) was concentrated using optimum method of urea complexation (ie., at 4 ^{o}C for 12h in 4:1 urea/fatty acid ratio) to obtain lipase hydrolyzed concentrated sardine fatty acids (LHCFA). LHCFA after converting to its methyl esters (LHCO) using BF_3/MeOH was further purified by optimum method of argentated-silica column chromatographic fractionation which used sequence of *n*-hexane:Me_2O to obtain 7 fractions. The fraction with maximum purity of EPA, DHA and total PUFA (fraction 6 using *n*-hexane: Me_2O 90:10) was designated as lipase hydrolyzed purified sardine methyl esters (LHPO).

5.1.5. The protocols of various analyses have been discussed in this section.

5.1.6. Statistical analysis: This section discussed the statistical analysis used in this chapter.

5.2. Results and Discussion

This section detailed the results and discussion of this chapter, which showed that the winterization of PUFA by Me_2O: EtOAc (7:3 v/v) treatment followed by urea complexation and argentation chromatography yielded a final purified PUFA (Total PUFA = 96.7%) rich in *n*-3 fatty acid methyl esters, such as EPA (47.6 %) and DHA (38.2 %). This procedure for concentrating *n*-3 PUFA esters, would contribute to the commercial application to obtain concentrated *n*-3 fatty acids, particularly EPA esters from sardine oil. The present study also showed the changes in different classes of fatty acid and composition as a function of time-course (1–6 h) of hydrolysis of triglycerides from sardine oil by a lipase purified from *Bacillus circulans* that may be a potential enzyme source for concentration of *n*-3 PUFAs of acyl chain length shorter than C20. It was found that ester bonds of n-3 fatty acid homologues were highly resistant to hydrolysis by lipase from *B. circulans,* resulting in their enrichment in fatty acid triglycerides. Structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic and steric properties to lipase-catalyzed hydrolytic effects on fatty acyl ester bonds of triglycerides. The fractions eluted with *n*-hexane/Me_2O (95:5, v/v) and *n*-hexane/EtOAc (9:4, v/v), by argentated chromatography furnished 20:5*n*-3 ester in final purities of 68.3 % of total fatty acids. A combination of *B. circulans* lipase-catalyzed hydrolysis followed by urea fractionation and argentated chromatography, is a promising method for separating and concentrating *n*-3 fatty acids such as 20:5*n*-3 from sardine oil.

CHAPTER 6: PREPARATION OF DIFFERENT NATURAL ADDITIVES TO STABILIZE SARDINE OIL

6.1. Materials and Methods

6.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals, reagents and analytical instruments used in this chapter

6.1.2. Samples and Study Area

This section detailed the samples and collection sites of different samples used in this chapter.

6.1.3. Preparation of Extracts from Natural Additives

6.1.3.1. Marine halophytes

This section demonstrated the preparation of ethyl acetate extracts of tropical halophytes, *viz*., *Salicornia brachiata* Miq., *Arthrocnemum indicum* Nels., *Suaeda maritima* L. Dumort, *Suaeda monoica* Forssk ex JF Gmel. from Chenopodiaceae; and *Sesuvium portulacastrum* L. from Azoaceae, abundantly available along the Southeast coast of the Indian subcontinent. This section also describes the evaluation of antioxidant activities of the halophyte extracts.

6.1.3.2. Marine Seaweeds

This section demonstrated the preparation of crude red seaweed extracts as follows: In brief, the powdered red seaweeds., *Kappahycus alvarezii*, *Gracilaria corticata*, *Hypnea muscifomis*, *Hypnea valentiae* and *Jania rubens* (100 g) were extracted with methanol (50 - 60 °C, 3 h), concentrated (50 °C) before being partitioned successively with *n*-hexane (150 mL×3), DCM (150 mL×3) and ethylacetate (150 mL×3), concentrated *in vacuo* to furnish *n*-hexane, dichloromethane, and ethylacetate fractions, respectively. This section also describes the evaluation of antioxidant activities of the seaweed extracts.

6.1.3.3. Natural herbal extracts

The fresh leaves of rosemary, *Rosmarinus officinalis*, leaves (50 g) were air dried, ground, and were subjected to soxhlet extraction (75-80^{0}C) using ethyl acetate. The pooled extract was filtered and concentrated to obtain ethyl acetate extract (5.6 g). The extract was then stirred with *n*-hexane (25 ml; 20 min), centrifuged and the supernatent was concentrated to obtain the refined extract of *R. officinalis*. The ground green tea, *Camellia sinensis*, leaves (10 g) were stirred with 50% aqueous ethanol (100 mL) at room temperature and filtered. The filtrate was mixed with an equal volume of chloroform, separated and the upper aqueous layer was extracted three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and concentrated to obtain the refined extract of *Camellia sinensis*.

6.1.4. Antioxidant Activity Assays

This section demonstrated the methodologies for various antioxidant actvivty assays as follows: 6.1.4.1. Determination of total phenolic compounds, 6.1.4.2. Free radical scavenging ability assays {6.1.4.2A. 1, 1-Diphenyl-2-Picryl- Hydrazil (DPPH•) radical

scavenging activity, 6.1.4.2B. 2, 2′-Azino-bis-3 Ethylbenzothiozoline-6- sulfonic acid Diammonium salt (ABTS) Radical Scavenging Activity}, 6.1.4.3. Hydroxyl ($HO^{\cdot}$) Radical Scavenging Activity, 6.1.4.4. Hydrogen Peroxide (H_2O_2) scavenging activity, 6.1.4.5. Lipid peroxidation inhibition activity in model system: Thiobarbituric Acid-Reactive Species (TBARS) formation inhibitory activity, 6.1.4.6. Reducing Ability and 6.1.4.7. Ferrous ion (Fe^{2+}) chelating activity

6.1.5. Statistical Analysis: This section detailed the methodologies adopted for statistical analyses.

6.2. Results and Discussion

This section detailed the results and discussion of this chapter, which provide information regarding the potential of halophytes and red seaweeds to develop natural sources for antioxidants as food supplements, as nutraceuticals and/or functional foods, and candidates in combating carcinogenesis and inflammatory diseases. The significant positive correlation of total phenolic content with different antioxidant activities indicate that phenolic principles present in these marine additives are endowed with potential antioxidant properties. A blend prepared from the EtOAc extracts of the halophytes, *S. brachiata* and *S. maritima* were observed to possess high antioxidant potential as compared to other halophytes when their *in vitro* antioxidant properties were considered. Similarly, the EtOAc fractions obtained from the crude methanolic extracts of the red seaweeds, *K. alvarezii*, *H. musciformis* and *J. rubens* revealed higher overall antioxidant potential as compared to *G. corticata* and *H. valentiae*. However, *H. valentiae* showed higher content of total phenolics as compared to *J. rubens* and *K. alvarezii* and the former it also showed better ABTS radical scavenging activity than that of *J. rubens.* Furthermore, *H. valentiae* also exhibited higher hydroxyl radical scavenging activity than *H. musciformis*. Similarly, *G. corticata* showed better ABTS radical scavenging activity as compared to *H. musciformis*. It is therefore, that these five candidate seaweeds along with the two halophytic plant extracts, *S. brachiata* and *S. maritima*, were selected to assess the effect of these marine natural additives on lipid oxidation in various forms of fish oil in the following chapter (*Chapter 7*).

CHAPTER 7: STABILITY STUDY OF SARDINE OIL

7.1. Materials and Methods

7.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals, reagents and instrumentation used in this chapter.

7.1.2. Experimental design of stability study

The different additives added to refined sardine oil (RO), concentrated sardine fatty acid methyl esters (CO) and purified sardine fatty acid methyl esters (PO) at 0.5% w/w were evaluated for stability studies. The synthetic additives, BHT and α- tocopherol (at 0.1% w/w) were used as controls in all the stability studies conducted. A schematic diagram of the stability study design is represented below:

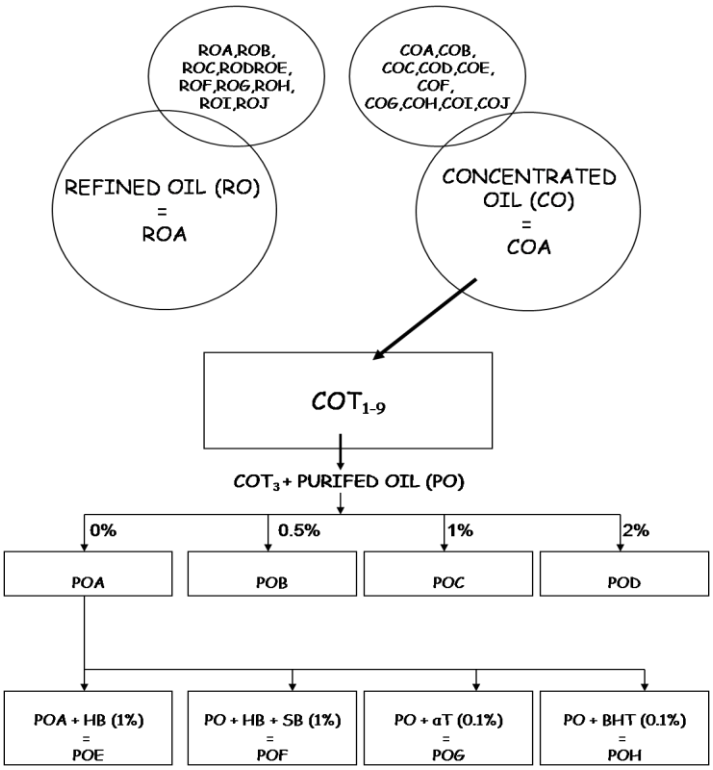


Fig. 7.1. A schematic diagram of the stability study design. The refined oil and concentrated methyl esters (ROA & COA) were added with 0.5% w/w of *S. brachiata* (ROB & COB), *S. maritima* (ROC & COC), *K. alvarezii* (ROD & COD), *G. corticata* (ROE & COE), *H. musciformis* (ROF & COF), *H. valentiae* (ROG & COG) and *J. rubens* (ROH & COH), α-tocopherol and BHT (at 0.1 % w/w; POI & POJ, respectively) and studied for OSI and accelerated stability at 65 ^{o}C. The potent additives shortlisted from this preliminary study were added in 9 factorial combinations to the concentrated methyl esters (COT_1- COT_9 as in Table 7.1) and further studied for OSI and accelerated stability at 65 ^{o}C. From these combinations, one potent combination was selected (ie. COT_3) and added to purified methyl esters at 0, 0.5, 1 and 2 % w/w concentration to afford POA, POB, POC and POD, respectively and studied for OSI and accelerated stability at 65 ^{o}C. The purified methyl esters was further added with herbal blend (1%; POE), herbal blend + COT_3 (0.5 + 0.5%; POF) and compared with α-tocopherol and BHT (at 0.1 % w/w) which used as positive controls (POG and POH) by studying the OSI and accelerated stability at 65 ^{o}C.

7.1.3. Assessment of oxidative stability of sardine oil/methyl esters by accelerated oxidation methods

The assessment of oxidative stability, which was performed using (7.1.3.1.) rancimat analysis and (7.1.3.2.) accelerated storage trial have been discussed in this section.

7.1.4. This section described the analytical methods used in this Chapter: (7.1.4.1.) DPPH activity of fish oil/esters and (7.1.4.2.) analysis of other oil quality parameters as in previous chapters

7.1.5. Statistical Analysis: This section detailed the methodologies adopted for statistical analyses.

7.2. Results and Discussion

This section detailed the results and discussion of this chapter, which demonstrated the efficacy of the seaweed extracts, mainly, *K. alvarezii*, *H. musciformis* and *J. rubens* for use as antioxidative supplements to the refined oil (EPA 14.7 %; DHA 5.7 %; ∑PUFA 25.8 %), concentrated (EPA 33.4 %; DHA 27.8 %; ∑PUFA 73.9 %) and purified fatty acid methyl esters (EPA 47.6 %; DHA 38.2 %; ∑PUFA 97.3 %) in a time dependent accelerated shelf life study (for 12 days) with respect to their oil stability indices (induction time & stability time), peroxide, para-anisidine, TOTOX and TBARS values, free radical scavenging activity along with the fatty acid composition. Both refined oil and fatty acid concentrate of sardines were demonstrated to be significantly protected by the *K. alvarezii*, *H. musciformis* and *J. rubens* extracts. The effect of the seaweed extract blend (especially, COT_3 prepared by mixing 0.1:0.2:0.2 % w/w of *K. alvarezii*, *H. musciformis* and *J. rubens* extracts, respectively) at 0.5 % level to the concentrated fatty acid methyl esters, showed a positive synergistic effect as calculated from its degree of synergism (% SYN 221.8 %). Furthermore, a threshold level of this blend COT_3 (1 % to purified fatty acid methyl esters) was found to be sufficient for optimum antioxidant activity, and further addition of this antioxidant blend (2 % to purified fatty acid methyl esters) does have no additional improvement on the stability of the essential long chain fatty acids. A significant reduction of *n*-3 PUFAs, and deficiency to arrest the isomeric conversion of *cis* PUFAs to their *trans* isomers of purified fatty acid methyl esters were realized, and this drawback was solved by the addition of the marine antioxidant combination. This combination further realized a comparable antioxidative effect as that of the herbal blend prepared from rosemary and

green tea extracts. The quality of the oil/esters was also further validated by ^{1}H NMR spectroscopy conjugated with FT-IR functional group analyses, which led us to infer that the antioxidative natural additives play a major role in retaining the native form of polyunsaturated fatty acids for a longer period on shelf. These additives also proved to prevent the formation of undesirable small molecular weight aldehydes and aromatic compounds from the fatty acid precursors on shelf. This study demonstrated the optimized protocol to maintain the sardine oil/esters stabilized with marine additive to retain their functional properties for a longer period on shelf.

CHAPTER 8: ISOLATION AND CHARACTERIZATION OF BIOACTIVE SECONDARY METABOLITES FROM POTENTIAL ADDITIVES

Background

The extracts of *Kappaphycus alvarezii*, *Hypnea muscifomis* and *Jania rubens* were found to possess properties to enhance the oxidative stability of the fish oil. This seaweed extracts imparted protection against lipid degradation in the refined, concentrated as well as purified sardine oil. Hence, the purification of these crude extracts was carried out by repeated purifications with the aid of various chromatographic techniques. The structural characterization was done with the aid of various spectroscopic techniques. The pure compounds were further assayed to document its free radical scavenging activity, lipid peroxidation inhibitory activity and Fe^{2+} ion chelating potential.

8.1. Materials and Methods

8.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals, reagents and instrumentation used in this chapter.

8.1.2. Isolation and characterization of bioactive secondary metabolites from potential additives

This section described the protocol of bioassay guided isolation of the antioxidant secondary metabolites from the potential marine flora ie. seaweeds, using different chromatographic techniques. The structural characterization of the purified compounds have been carried out with the aid of various spectroscopic techniques such as FTIR, GC-

MS and NMR including 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HMBC, HSQC and NOESY) experiments. The species of red seaweeds were *Kappaphycus alvarezii* (section 8.1.3.1), *Hypnea muscifomis* (8.1.3.2) and *Jania rubens* (8.1.3.3).

8.1.2.1. Bioassay guided chromatographic purification of the *Kappaphycus alvarezii*

The EtOAc fraction of the MeOH extract of *K. alvarezii* was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*-hexane, EtOAc:MeOH and finally MeOH to obtain different fractions. The 6 fractions, KA_3, KA_5, KA_6, KA_7, KA_8 and KA_9 were further chromatographed using silica column/ silica preparative thin layer chromatographic techniques.

8.1.2.2. Bioassay guided chromatographic purification of the *Hypnea muscifomis*

The EtOAc fraction of the MeOH extract of *H. muscifomis* was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*-hexane, EtOAc:MeOH and finally MeOH to obtain different fractions. The 4 fractions, HM_1, HM_2, HM_4 and HM_7 were further chromatographed using silica column/ silica preparative thin layer chromatographic techniques.

8.1.2.3. Bioassay guided chromatographic purification of the *Jania rubens*

The EtOAc fraction of the MeOH extract of *J. rubens* was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*-hexane, EtOAc:MeOH and finally MeOH to obtain different fractions. The 3 fractions, JR_6, JR_8 and JR_9 were further chromatographed using silica column/ silica preparative thin layer chromatographic techniques.

8.1.3. Analysis

The protocols for analyzing the UV absorption, GC-MS, LC-MS, FTIR and detailed NMR analyses have been discussed in this section.

8.1.4. Statistical analysis: This section discussed the statistical analysis used in this chapter.

8.2. Results and Discussion

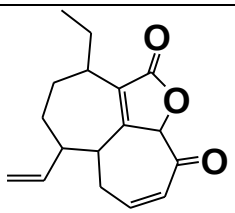
8.2.1. Secondary Metabolites from *Kappaphycus alvarezii*

8.2.1.1. Structural Characterization of Compound I (KA-36)

The compounds with potential antioxidative activity isolated from *Kappaphycus alvarezii* are (8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione which possessed 81.1 % DPPH radical scavenging activity, 0.99 TBARS formation inhibitory activity and 3.11 % Fe^{2+} ion chelating activity.

Another compound, (2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1, 4, 5, 5a, 6, 9, 10, 10a-octahydro-1- heptalenecarboxylate., isolated from *K. alvarezii* showed 69.1 % DPPH radical scavenging activity, 0.11 MDAEQ/kg TBARS formation inhibitory activity and 41.0 % Fe^{2+} ion chelating activity.

COMPOUND I (KA-36)

(8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione	
	
Sample yield	25 mg (2.08 %*)
Physical description	Greenish oil
Molecular formula	$C_{17}H_{20}O_3$
Molecular weight	272.4568

* Yield with respect to EtOAc fraction

The pure active COMPOUND I (KA-36) was found to be as (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione.

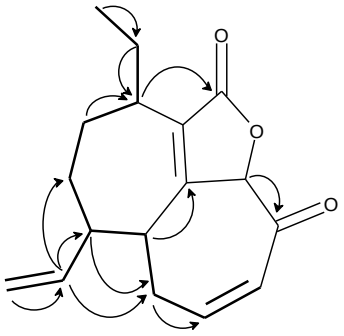
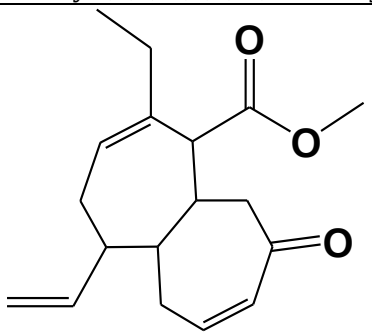


Fig. 8.5.1 . ^{1}H–^{1}H COSY and HMBC correlations of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

8.2.1.2. Structural Characterization of Compound II (KA-37)

COMPOUND II (KA-37)

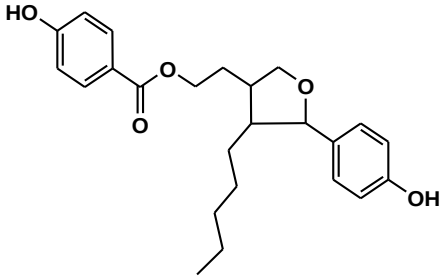
(2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1- heptalenecarboxylate	
	
Sample yield	61.5 mg (5.1 %*)
Physical description	Yellow powder
Molecular formula	$C_{18}H_{24}O_3$
Molecular weight	288.3956

* yield with respect to EtOAc fraction

8.2.2. Secondary Metabolites from *Hypnea musciformis*

The compounds isolated and characterized from *Hypnea muscifomis* are 2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl)ethyl 4-hydroxybenzoate, 2-2-[(4-hydroxybenzoyl)oxy]ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2H-6-pyranylbutanoic acid, and 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate which possessed > 49 % DPPH radical scavenging activity. Among these 2-[4-butyl-5-(4-hydroxyphenyl) tetrahydro-3-furanyl] ethyl 4-hydroxybenzoate showed high Fe^{2+} ion chelating activity (40.2 %).

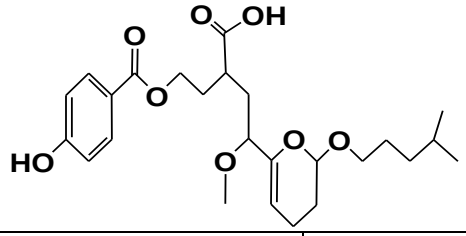
Compound I (HM-25)

2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl)ethyl 4-hydroxybenzoate	
	
Sample yield	126.8 mg (1.58 %*)
Physical description	White semisolid
Molecular formula	$C_{24}H_{30}O_5$
Molecular weight	398.6984

* % yield based on starting EtOAc extract

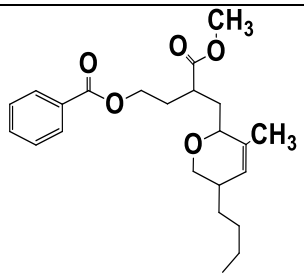
8.2.2.2. Structural Characterization of Compound II (HM-45)

Compound II (HM-45)

2-2-[(4-Hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2H-6-pyranylbutanoic acid	
	
Sample yield	50.5 mg (0.63 %*)
Physical description	Yellow semi-solid
Molecular formula	$C_{25}H_{36}O_8$
Molecular weight	464.4268

* % yield based on starting extract

8.2.2.3. Structural Characterization of Compound III (HM-50)

Compound III (HM-50)	
3-((5-Butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate	
	
Sample yield	81.44 mg (1.02 %*)
Physical description	Pale yellow powder
Molecular formula	$C_{23}H_{32}O_5$
Molecular weight	388.4814

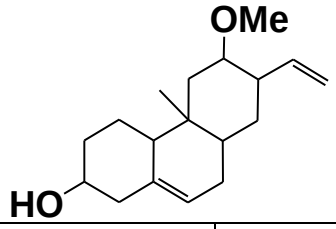
* % Yield based on starting extract

8.2.3. Secondary Metabolites from *Jania rubens*

8.2.3.1 Structural Characterization of Compound I (JR -17)

The compound 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol isolated from the red seaweed *Jania rubens* was found to possess potential antioxidative properties with 91.4 % DPPH radical scavenging activity, 0.88 MDAEQ/kg TBARS formation inhibitory activity and 40.4 % Fe^{2+} ion chelating activity. The chemical structures of the pure compounds isolated with higher yield, as well as their relative stereochemistries, were established by means of spectral data analyses, including 2D-NMR experiments.

Compound I (JR -17)

6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol	
	
Sample yield	45.65 mg (0.57 %*)
Physical description	Yellow oil
Molecular formula	$C_{18}H_{28}O_2$
Molecular weight	276.4862

* % yield based on starting EtOAc fraction

CHAPTER 9: PREPARATION OF CEPHALOPOD EXTRACT FOR THE ENRICHMENT OF PURIFIED SARDINE OIL

9.1. Materials and Methods

9.1.1. Chemicals, Reagents and Instrumentation

This section detailed the chemicals, reagents and instrumentation used in this chapter.

9.1.2. Samples and Study Area

This section detailed about the samples of cephalopods used in this chapter.and their collection area

9.1.3. Preparation of Cephalopod Extracts

This section detailed the preparation of the extracts from the muscle tissues of cephalopods, *Loligo duvaucelli*, *Sepia officinalis*, *Octopus membranaceus* and *Cystopus indicus* with EtOAc: MeOH (1:1, v/v). These extracts were studied for *in vitro* antioxidant and anti-inflammatory activities and the potent extract., ie. *Loligo duvaucelli* extract was selected for purification and structural characterization.

9.1.4. *In vitro* Anti-Inflammatory Activities of the Cephalopod Extracts

This section details the procedure for evaluating the *in vitro* anti-inflammatory activities of the cephalopod extracts using (9.1.4.1.) Cyclooxygenase-1&2 (COX-1&2) inhibition and (9.1.4.2.) Lipoxygenase-5 (LOX-5) inhibition assays

9.1.5. Fatty acid composition of *Loligo duvauceli*

This section details the procedure for evaluating fatty acid composition of *Loligo duvauceli*

9.1.6. *In vivo* Anti-inflammatory Activity of the *Loligo duvauceli* Extract

This section details the procedure for *in vivo* carrageenan-induced mice paw edema experiment of *Loligo duvauceli extract*

9.1.7. Purification of *Loligo duvaucelli*

This section described the protocol of bioassay guided isolation of the antioxidant secondary metabolites from the solvent extract of *Loligo duvaucelli* using pluralities of chromatographic techniques.

9.1.8. DPPH Radical Scavenging Activity

This section detailed the methodologies adopted for DPPH Radical Scavenging Activity of the extracts and purified fractions

9.1.9. Other Analyses

This section described other analytical methods used in this Chapter.

9.1.10. Statistical Analysis

This section detailed the methodologies adopted for statistical analyses.

9.2. Results and Discussion

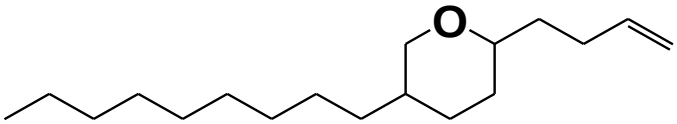
This section detailed the results and discussion of this chapter which showed that among the cephalopods studied for anti-inflammatory properties, *Loligo duvaucelli* exhibited significantly higher ability to inhibit the COX-2 and 5-LOX enzymes which included in inflammation processes. The *L. duvaucelli* extract also showed reduction in paw edema of the carragenan induced mice in an *in vivo* anti-inflammatory study. *L. duvaucelli* extract, when added to purified FAMEs showed maximum anti-inflammatory potential at 0.5% concentration.The *in vivo* anti-inflammatory properties of *Loligo duvaucelli* showed to be persisted throughout the period of study, the activities improved with time, indicating their potential to release the active principle throughout an extended period.

The purification and structural characterization of *Loligo duvauceli* derived the compounds belonging to pyran derivatives such as 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran and 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran and were demonstrated to possess potential anti-inflammatory and antioxidative properties. 2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran (Compound I) showed 37.4 % COX-2 inhibition activity, 33.01 % LOX-5 inhibition activity (both at 0.5mg/ml) with 68.6 % free radical scavenging activity. The compound II, exhibited 36.2 % COX-2 inhibition activity, 30.1 % LOX-5 inhibition activity (both at 0.5mg/ml) with 71.7 % free radical scavenging activity. The anti-inflammatory potential of these compounds derived from *Loligo duvauceli* are proved to be comparable to the synthetic anti-inflammatory agents, aspirin and indomethacin. The compound are shown as follows:

9.2.4.1. Structural Characterization of Compound I (LD-32)

Compound I (LD-32)

2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran



Sample yield	65 mg (0.33 %*)
Physical description	Pale yellow powder
Molecular formula	$C_{18}H_{34}O$
Molecular weight	266.4752

* % yield based on starting *Loligo duvaucelli* extract

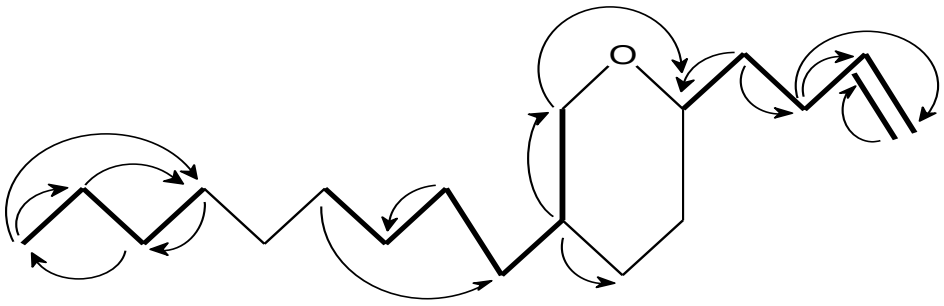
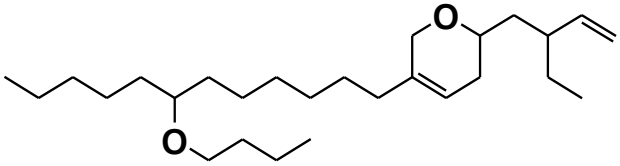


Fig. 9.5.1. $^{1}H–^{1}H$ COSY and HMBC correlations of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

9.2.4.2. Structural characterization of COMPOUND II (LD - 56)

COMPOUND II (LD-56)

5-(7-Butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2H-pyran	
	
Sample yield	84 mg (0.42 %*)
Physical description	White semi-solid
Molecular formula	$C_{27}H_{50}O_2$
Molecular weight	406.7592

* % yield based on starting *Loligo duvaucelli* extract

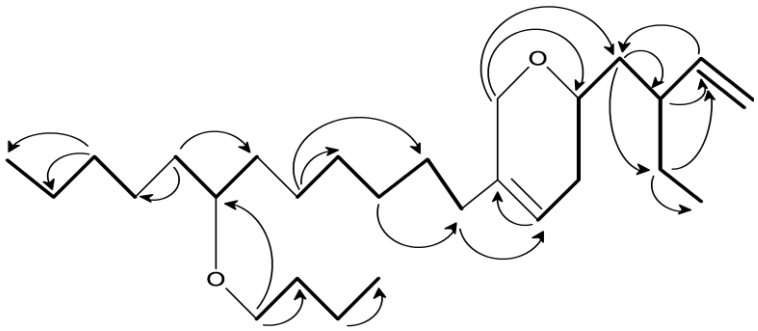


Fig. 9.6.1. ^{1}H–^{1}H COSY and HMBC correlations of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

CHAPTER 10: PREPARATION OF ENRICHED PUFA FORMULATION FOR USE AS NUTRACEUTICAL AND AQUAFEED SUPPLEMENTS

10.1. Materials and Methods

10.1.1. This section detailed the chemicals, reagents and instrumentation used in the chapter

10.1.2. Preparation of an Enriched Anti-Inflammatory PUFA Formulation from Purified Sardine Oil

The purified sardine methyl esters (PO) with the antioxidant combination of the red seaweed extracts of *K. alvarezzi*, *H. musciformis* and *J. rubens* at 1% (POC) was blended with *Loligo duvauceli* extract (0.5%, w/w) and designated as POI. These were studied for their (10.1.3) *in vivo* anti-Inflammatory activity.

10.1.3. *In vivo* Anti-Inflammatory Activity

This section details the procedure for *in vivo* carrageenan-induced mice paw edema experiment of purified sardine fatty acid esters and those supplemented with additives.

10.2. Results and Discussion

10.2.1. Enrichment of Purified Sardine Fatty Acid Methyl Esters for use as Nutraceutical Supplements

The addition of *Loligo duvauceli* significantly enhanced the anti-inflammatory property of the PUFA enriched purified oil, which has been demonstrated by the carrageenan-induced paw edema model study in mammalian subjects. The anti-inflammatory activity of the PUFA enriched formulation was found to be significantly higher after 4 h of carrageenan injection. It is therefore, the PUFA enriched sardine oil supplemented with *Loligo duvauceli* extract may be used as a nutraceutical supplement human health and nutrition, and is an effective alternative to the synthetic non steroidal anti-inflammatory drugs (NSAIDs) for use against mammalian inflammatory diseases. It is evident from the earlier chapter (*Chapter 7*) that the purified methyl esters supplemented with marine antioxidants were able to retain the stability of long chain polyunsaturated fatty acids, especially, EPA and DHA. It is therefore, that the stabilized purified methyl esters prepared in the present work can be used as aquafeed supplement, and could be able to maintain the palatability, odor and health benefits of the aquafeed for a prolonged period.

CHAPTER 11: SUMMARY

A summary of entire work has been detailed in this chapter.

REFERENCES

The references from all the chapters have been cited in this section

PUBLICATIONS

The list of published and accepted publications from the thesis work is detailed in this section

Dr. Kajal Chakraborty
(Research Guide)

Deepu Joseph
(Research Student).